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Molecular phylogenetics of sexual and parthenogenetic *Timema* walking-sticks

C. Sandoval[1], **D. A. Carmean**[2] **and B. J. Crespi**[2]

[1]*Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, CA 93106, USA* (sandoval@lifesci.ucsb.edu)
[2]*Department of Biosciences, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6*

We inferred a phylogeny for the walking-stick genus *Timema* (Insecta: Phasmatoptera) using mitochondrial DNA sequence, and we used the phylogeny to infer temporal patterns of speciation and the evolutionary history of parthenogenesis. Maximum parsimony, neighbour-joining and maximum-likelihood analyses of 660 base pairs (bp) of cytochrome oxidase I (*COI*) yielded phylogenies that were well resolved and topologically identical or very similar. Application of an insect molecular clock for *COI* suggests that the genus originated in southern California, northern Mexico or Arizona about 20 million years ago and underwent a burst of speciation 1.5–3 million years ago during the uplifts of the Sierra Nevada, Coast, and Transverse Ranges. The phylogeny indicates that the three parthenogenetic lineages of *Timema* have arisen independently and are each closely related to morphologically indistinguishable or similar sexual species. Each of the three lineages exhibits an allopatric or parapatric, and more northerly, distribution with regard to their closest sexual relative. *COI* divergence levels between each of the three parthenogens and their closest sexual relative suggest ancient origins of parthenogenesis, 1.5–3 million years ago, that may coincide with the extensive glaciation that formed the North American ice sheets.

Keywords: parthenogenesis; speciation; walking-sticks

1. INTRODUCTION

Robust evaluation of alternative hypotheses for the evolution of sex and asexuality requires integration of phylogenetic, biogeographic, ecological and genetic data (Bell 1982). Hypotheses concerning the phylogenetic history and age of asexual taxa have been especially controversial (Judson & Normark 1996; Little & Hebert 1996), due to the paucity of reliable phylogenetic data for clades with sexual and asexual species and the difficulty of inferring dates of speciation events. Ancient asexual taxa, should they exist, may yield critical clues to understanding the causes of the evolution of sex, as aspects of their genetics and ecology presumably differ from shorter-lived asexuals in ways that indicate the selective advantages of asexual and sexual systems (Judson & Normark 1996).

The walking-stick genus *Timema* (Insecta: Phasmatoptera: Timemidae) provides an excellent opportunity to study the evolutionary history of sex and asexuality because 3 of the 13 described *Timema* species are parthenogenetic (Vickery 1993; Sandoval & Vickery 1996). This high incidence of asexuality allows for multiple comparisons of asexual lineages with their sexual relatives, within a small clade whose ecology has been the subject of considerable study (Sandoval 1994*a*,*b*). In this paper, we infer a phylogeny for all 13 described species of *Timema* using mitochondrial DNA (mtDNA) sequence. We use the phylogeny to investigate the evolutionary and biogeographic history of the genus, and the ages of the three described taxa that are parthenogenetic.

2. MATERIALS AND METHODS

(a) *Natural history and taxonomic background*

The genus *Timema* comprises 13 described species that are restricted to the mountains in western North America between 30° and 42° N (Vickery 1993). These walking-sticks are herbivores and primarily inhabit host-plants in chaparral vegetation (Vickery 1993). They rest on branches or leaves of vegetation during the day and feed at night, relying on crypsis for protection against predators; their colour pattern is fine-tuned to match the colour pattern of their host-plants, and some species exhibit host-associated colour polymorphisms (Sandoval 1994*a*,*b*; C. Sandoval, unpublished data).

The taxonomy of *Timema* species is based on external morphological characters, particularly the male genitalia. Consequently, there has been confusion on the identification of the all-female parthenogenetic species (Sandoval & Vickery 1996). Parthenogenesis has been documented in three taxa: *T. tahoe*, *T. genevieve* and *T. douglasi* (Rentz 1978; Vickery 1993; Sandoval & Vickery 1996). The parthenogenetic status of these species was confirmed via an absence of males in the field and production of viable offspring by unmated females in the laboratory (Sandoval & Vickery 1996; C. Sandoval, unpublished data).

Timema tahoe and *T. genevieve* each has a morphologically indistinguishable sexual relative, *T. bartmani* and *T. podura*, respectively, and is found on the same host-plant species as its sexual relative (C. Sandoval, unpublished data). By contrast, *T. douglasi* closely resembles *T. californicum* morphologically, except that it exhibits a different colour pattern that matches its different host (Sandoval & Vickery 1996). The status of *Timema* parthenogens as automictic or apomictic is unknown; both automixis or apomixis have been

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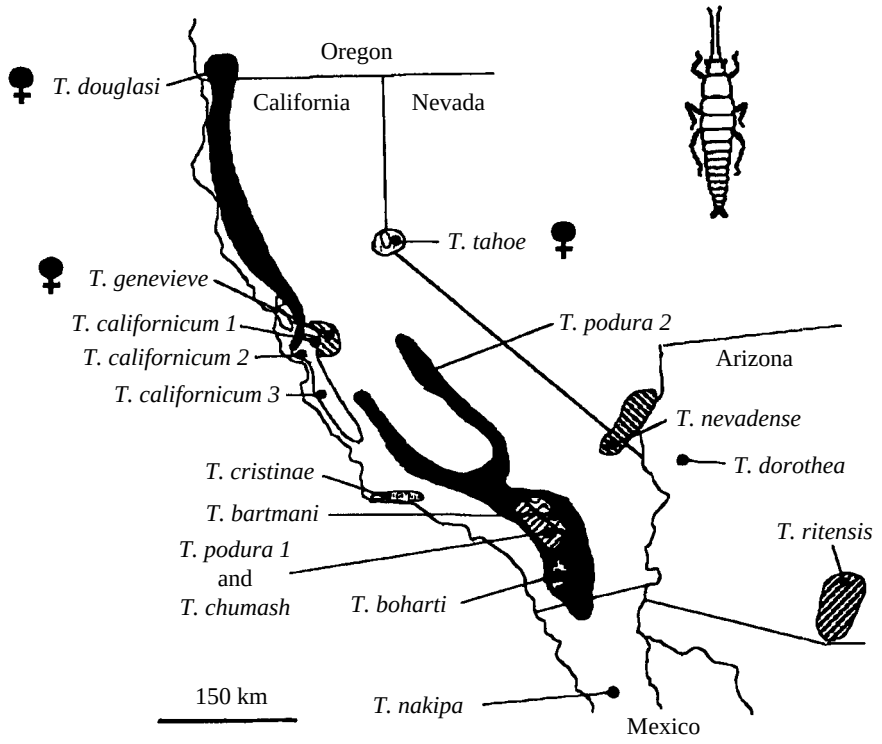

Figure 1. Map showing areas of distribution of each *Timema* species based on museum records listed in Vickery (1993) and observations by the authors. The ranges of *T. dorothea* and *T. nakipa* are too small for the scale of the map. The collection sites of the taxa for this study are marked with dots. Parthenogenetic species are marked with a female symbol.

reported for other representatives of the Phasmatoptera (Marescalchi *et al.* 1991, 1993). Laboratory experiments indicate that neither parthenogenetic females of *T. douglasi* nor *T. genevieve* will mate with males of their sexual relative (C. Sandoval, unpublished data); phenological separation of adults of *T. tahoe* and *T. bartmani* precludes mating tests with these taxa.

(b) *Geographic distributions*

Timema species have been collected from the northen Baja Peninsula, east to Arizona, and north to about 42° N (figure 1). The southernmost distribution of two parthenogenetic species, *T. douglasi* and *T. genevieve*, touches the northernmost distribution of their sexual counterparts. By contrast, a large geographic gap, which may be filled through additional collecting effort, isolates *T. tahoe* from *T. bartmani*. Further collecting should expand the documented distributions of most other species, at least on a local geographic scale.

(c) *Collection*

Specimens of *Timema* were collected by shaking branches of host vegetation inside of a large sweep net. Immature individuals were raised in the laboratory until maturity for identification by Vernon Vickery (Lyman Entomological Museum and Research Laboratory). Voucher specimens have been deposited at the California Academy of Sciences, San Francisco, CA, and in the personal collection of C. Sandoval. Except for *T. dorothea*, for which we could only obtain three specimens, all field collections yielded at least 20 individuals. *Timema podura* was collected from two allopatric populations, and *T. californicum* was collected from three allopatric populations (figure 1).

(d) *Mitochondrial DNA data*

DNA was isolated from live or frozen specimens by crushing with a sterilized glass pipette in Lifton buffer (0.2 M sucrose, 0.05 M EDTA, 0.1 M Tris, 0.5% SDS), extraction using phenol-chloroform-isoamyl alcohol and precipitation in 70% ethanol with 0.7 M sodium acetate. Double-stranded PCR amplifications were done using the primer pairs S2183–A3014, S2195–A3014, S2183–A2887, and S2195–A2887 (Simon *et al.* 1994), which amplify most of the 3′ half of the cytochrome oxidase I (*COI*) gene. Double-stranded products were digested with exonuclease I and shrimp alkaline phosphatase to inactivate free nucleotide and primer, and the double-stranded DNA was sequenced directly in both directions using dideoxy sequencing with Sequenase and S35 label. Sequences have been deposited in GenBank under accession numbers AF005330–AF005347.

(e) *Phylogenetic inference*

The order Phasmatoptera (the stick insects) comprises two families, the Timemidae and the Phasmatidae, and Kristensen (1975) suggested that *Timema* and Phasmatidae are sister taxa. We therefore used as outgroups two species of Phasmatidae, *Baculum extradentatum* and *Anisomorpha buprestoidea*. Subsequent analysis showed that resolution of basal nodes was improved by addition of another outgroup, the cockroach *Blatella germanica* (Martinez-Gonzalez & Hegardt 1994). Cockroaches and stick insects are both in the infraclass Neoptera, division Polyneoptera (the orthopteroids; Gillot 1980).

We analysed the data using maximum-parsimony (branch-and-bound searches in PAUP 3.1.1; Swofford 1993), maximum-likelihood (PAUP* beta test version d59, written by D. L. Swofford), using the HKY substitution model with rate heterogeneity partitioning the data set into first, second and third codon sites, and neighbour-joining (DNADIST and NJBOOT in PHYLIP 3.5, using the Kimura two-parameter correction for multiple substitutions) methods. These multiple forms of analysis allow for evaluation of phylogeny robustness with respect to assumptions of phylogenetic methods.

(f) *Analyses of macroevolutionary patterns*

Using our phylogeny, we analysed (i) inferred dates at nodes (i.e. speciation events) on the tree, and (ii) inferred ages of origins of parthenogenesis.

To infer dates at nodes of the tree, we used molecular clock calibrations for arthropod mtDNA. A clock for arthropod mtDNA indicates rates of pairwise divergence of about 2% per million years (Ma) for taxa that diverged less than about 3 Ma ago (Brower 1994; Juan *et al.* 1995, 1996). After this time, the relationship between time and divergence increases at a decreasing rate, such that divergences of 8–10% correspond to about 5 Ma, 13–15% correspond to about 10 Ma, 16–18% correspond to about 15 Ma and 20% correspond to about 20 Ma (Juan *et al.* (1995, 1996) for *COI* in beetles). The ages of mountain ranges provide an approximate upper bound for inferred datings, because *Timema* are restricted to mountains and presumably could not have invaded particular regions prior to uplift. We tested the validity of our molecular clock assumption by comparing the likelihood of the best rate-constant tree with the likelihood of an unconstrained tree with the same topology (M. Berbee and J. Felsenstein, personal communication), using maximum likelihood in PAUP* beta test version d59 (as described above) and the Kishino–Hasegawa test.

3. RESULTS

(a) *Mitochondrial DNA data*

The *COI* data set comprised 660 base pairs, 236 of which were cladistically informative and 182 of which were cladistically informative in the ingroup. The majority (86%) of informative sites in the ingroup were third codon positions, and only 23 of the codons exhibited one or more amino acid substitutions.

Pairwise distances ranged from 0.8% to about 20% in the ingroup (table 1). Each of the three parthenogenetic species exhibited low levels of divergence from its morphologically indistinguishable or most closely similar sexual relative: *T. tahoe* differed by 3.3% from *T. bartmani*, *T. genevieve* differed by 4.5% from *T. podura* 2 (Sequoia National Park) and *T. douglasi* differed by 5.2%, 5.3% and 5.6% from three specimens of *T. californicum*. The three specimens of *T. californicum* showed pairwise divergences of 0.3%, 4.5% and 5.2%, which indicates that intraspecific divergences approach, and may even sometimes exceed, interspecific divergences in at least some *Timema* species.

(b) *Phylogenetic inference*

Maximum parsimony analysis of the full data set yielded four trees of length 805 (CI = 0.546, RI = 0.602). The majority-rule consensus of these trees indicated three nodes that varied between the four trees (figure 2*a*). The bootstrap majority-rule consensus tree was identical in topology to the majority-rule consensus tree except for the relationships of *T. podura* 1 (San Jacinto), *T. chumash* and *T. tahoe* (figure 2*a*). Bootstrap proportions were generally high (68–100%), except for six nodes that varied between 42 and 56%.

Maximum likelihood analysis gave a best tree that was identical in topology to the majority-rule consensus tree inferred using maximum parsimony. The ln likelihood of the maximum likelihood tree (−3121.3, all ingroup taxa included in the analysis) differed significantly from a tree with the same topology that was constrained to the rate-constancy model (−3141.7, difference in ln likelihoods = 20.4, s.d. = 6.8, $p < 0.05$), which indicates that a hypothesis of rate constancy along every lineage (a molecular clock) can be rejected for the full data set. We diagnosed the location of this rate variation by inspection of branch lengths under the constrained and unconstrained models, and found that the exclusion of three taxa, *T. cristinae*, *T. nakipa* and one sample of *T. californicum*, resulted in a lack of rejection of the clock (ln likelihood without clock = −2657.6, with clock = −2663.7, difference = 6.1, s.d. = 3.3, $p > 0.05$). Thus, with these three taxa excluded, inferences based on a clock can be justified statistically. This rate-constant maximum likelihood tree (figure 3) differed slightly in topology from the maximum parsimony and maximum likelihood tree (with all taxa included), with regard to several nodes exhibiting low bootstrap support.

The neighbour-joining tree was likewise identical to the majority-rule consensus tree inferred using maximum parsimony, except that *T. nevadense* did not form a monophyletic group with *T. dorothea* and *T. ritensis* (figure 2*b*). Neighbour-joining bootstrap analysis yielded bootstrap proportions that were notably higher than those for the maximum parsimony analysis.

Timema californicum specimens from three sites formed a monophyletic group in all trees inferred from the full data set, but *T. podura* from two sites was always polyphyletic. This polyphyly may be due to faster morphological divergence among lineages of a widespread ancestral species when speciation occurred in association with host shifts. For example, derivation of *T. chumash* from the ancestor of *T. podura* 1 involved a host shift that engendered morphological and reproductive divergence; by contrast, allopatric populations of *T. podura* that have not switched hosts have not diverged nor speciated.

(c) *Analyses of macroevolutionary patterns*

Our testing and use of a molecular clock calibration for *COI*, coupled with the results of the maximum likelihood analysis assuming rate constancy (with three taxa excluded, to satisfy the rate-constancy model), allows for inference of divergence times between *Timema* lineages (figure 3). The deepest pairwise divergences of about 20% correspond to an origin of the genus about 20 Ma ago. Because all four of the most-basal splits leading to extant species on our rate-constant maximum likelihood tree involve species with current distributions in southern California or Arizona (i.e. *T. ritensis*, *T. dorothea*, *T. nevadense* and *T. boharti*), we infer that the genus arose in the southern part of its current distribution. The tree also exhibits an apparent burst of speciation about 1.5–3 Ma ago, corresponding to about 3–6% pairwise divergence (table 1 and figure 3).

Our phylogenetic analyses, coupled with our knowledge of morphology and biogeography, indicates that parthenogenesis arose three times in *Timema*, once for each of the described parthenogenetic species. In each of the three cases, the parthenogenetic species is closely related to its morphologically indistinguishable or morphologically most-similar sexual relative: *T. douglasi* is sister-taxon to *T. californicum*, *T genevieve* is sister-taxon to *T. podura* 2, and *T. tahoe* is adjacent to (or otherwise very close to) *T. bartmani*. The ages of the three parthenogenetic lineages, inferred

Table 1. *Pairwise distances (per cent divergence) between taxa*

(Collection sites for intraspecific samples: *T. podura*, 1=San Jacinto, 2=Sequoia National Park; *T. californicum*, 1=Mount Hamilton, 2=Loma Prieta, 3=Carmel Valley.)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. *T. podura* 1																		
2. *T. podura* 2	0.068																	
3. *T. chumash*	0.035	0.040																
4. *T. tahoe*	0.059	0.053	0.027															
5. *T. bartmani*	0.052	0.042	0.027	0.033														
6. *T. genevieve*	0.067	0.045	0.047	0.052	0.040													
7. *T. boharti*	0.111	0.100	0.093	0.107	0.095	0.106												
8. *T. nakipa*	0.142	0.120	0.122	0.122	0.120	0.118	0.125											
9. *T. dorothea*	0.186	0.181	0.178	0.168	0.175	0.180	0.174	0.153										
10. *T. nevadense*	0.182	0.175	0.171	0.165	0.173	0.179	0.170	0.179	0.170									
11. *T. ritensis*	0.165	0.170	0.147	0.155	0.155	0.163	0.173	0.147	0.135	0.175								
12. *T. californicum* 1	0.187	0.176	0.176	0.175	0.185	0.179	0.173	0.153	0.159	0.190	0.162							
13. *T. californicum* 2	0.185	0.172	0.178	0.178	0.184	0.179	0.176	0.150	0.163	0.190	0.163	0.008						
14. *T. californicum* 3	0.186	0.174	0.173	0.181	0.179	0.183	0.179	0.162	0.180	0.181	0.165	0.052	0.045					
15. *T. douglasi*	0.183	0.172	0.165	0.170	0.170	0.176	0.172	0.150	0.162	0.181	0.164	0.056	0.053	0.053				
16. *T. cristinae*	0.177	0.173	0.172	0.173	0.166	0.176	0.192	0.152	0.171	0.186	0.149	0.144	0.141	0.144	0.133			
17. *Baculum*	0.237	0.238	0.236	0.239	0.239	0.234	0.240	0.225	0.229	0.243	0.237	0.233	0.227	0.235	0.230	0.213		
18. *Anisomorpha*	0.243	0.246	0.230	0.234	0.232	0.233	0.253	0.230	0.230	0.234	0.216	0.227	0.224	0.231	0.227	0.216	0.141	
19. *Blatella* (roach)	0.197	0.194	0.194	0.186	0.192	0.194	0.201	0.175	0.194	0.216	0.195	0.190	0.191	0.191	0.193	0.184	0.199	0.195

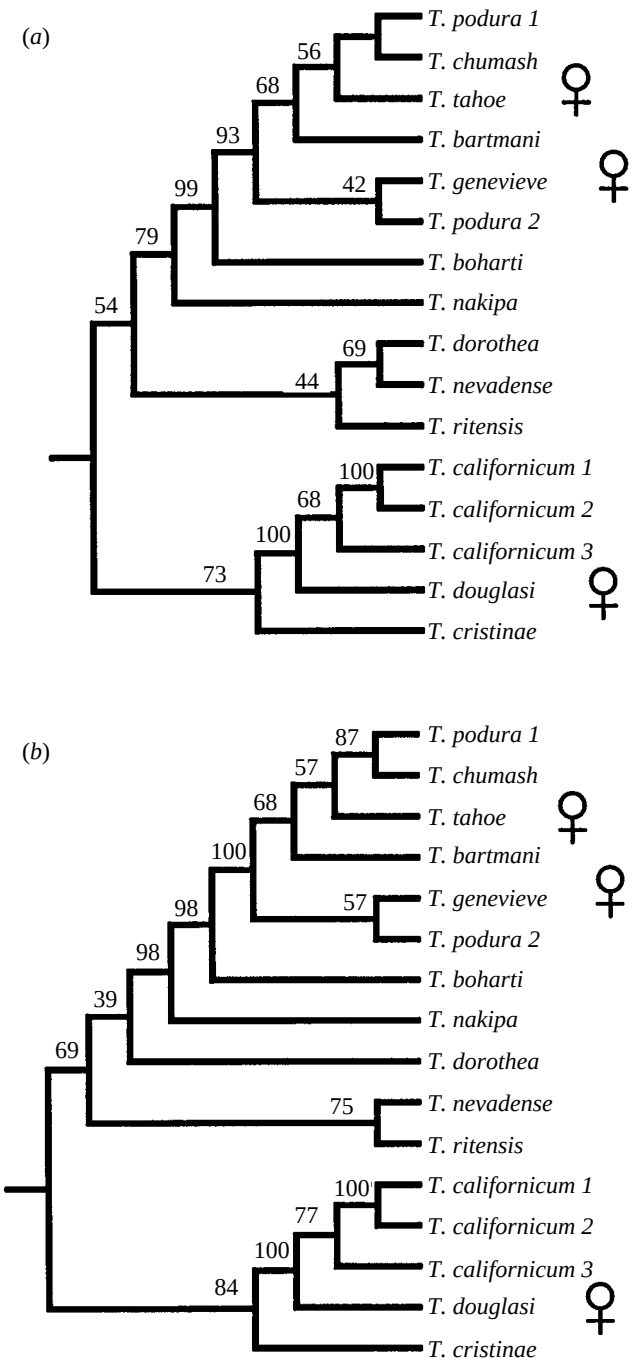

Figure 2. (*a*) Topology of the 50% majority-rule consensus of four trees of length 805 inferred using maximum parsimony, with bootstrap proportions from the bootstrap majority-rule consensus tree (500 replicates) shown above the branches. All majority-rule consensus proportions for the four trees (not shown) are 100%, except (*T. podura* 2 + *T. genevieve*): 50% and (*T. podura* 1 + *T. chumash*): 75%. This tree has the same topology as the maximum likelihood tree. The bootstrap majority-rule consensus tree differs slightly from the majority-rule consensus of the four shortest trees, in that it shows the relation ((*T. chumash*, *T. tahoe*), *T. podura*) rather than (*T. chumash*, (*T. tahoe*, *T. podura*)). *T. chumash* and *T. tahoe* are joined in the bootstrap majority-rule consensus tree with a bootstrap proportion of 42%. (*b*) Neighbour-joining tree, showing bootstrap proportions from 1000 replicates. In both (*a*) and (*b*), parthenogenetic species are marked with a female symbol.

from our *COI* clock and the rate-constant maximum likelihood tree, are similar to one another, between about 1.5 and 3 Ma old (figure 3). The parthenogens and their relatives exhibit a clear pattern of geographic parthenogenesis, with distributions that are further north and parapatric (*T. douglasi* and *T. genevieve*) or allopatric (*T. tahoe*) with respect to sexual relatives (figure 1).

4. DISCUSSION

Agreement between the tree topologies inferred from maximum parsimony, neighbour joining and maximum likelihood analyses, and the generally high maximum-parsimony and neighbour-joining bootstrap values, indicate that our phylogeny provides a good estimate of the evolutionary history of *Timema*. Uncertainty regarding the tree topology was restricted primarily to the placement of *T. nevadense*, and to particularities of the relationships between *T. podura*, *T. chumash*, *T. genevieve* and *T. tahoe*, although for the neighbour-joining tree the branching order of these taxa appeared reasonably well supported. Our finding that the overwhelming majority of nucleotide differences between *Timema* species involve synonymous substitutions indicates that for the species that have diverged within the past few million years, a pairwise divergence rate of about 2% per million years should provide an accurate estimate of divergence times (Brower 1994; Juan *et al.* 1995, 1996).

Our phylogeny, coupled with data on geographic distributions, rates of molecular evolution, and the timing of geological and palaeobotanical events, provides a simple scenario for the origin and diversification of *Timema* species. We have inferred from current distributions, our tree topology and levels of *COI* divergence that the genus originated about 20 Ma ago in the southern part of its current distribution. Although the dating of such an ancient event with an mtDNA clock is subject to considerable uncertainty, this dating and general location coincide well with the time and place for origin of the chaparral vegetation biome to which *Timema* are adapted (Axelrod 1986). The primary inferred direction of expansion in distribution of the genus, west and north, follows the distribution changes of chaparral over time (Axelrod 1980, 1986). Basal speciation events in the *Timema* are concentrated among the Arizona species *T. dorothea*, *T. nevadense* and *T. ritensis*, which are consistent with the estimated ages of formation of the Arizona mountain ranges 5–20 Ma ago (E. R. Force and D. Miller, personal communications).

Diversification of *Timema* is characterized by an apparent burst of speciation 1.5–3 Ma ago, during which time about half of the inferred speciation events took place. This radiation coincides with the dominant periods of uplift for the Sierra Nevada, Coastal and Tranverse ranges (Wahrhaftig & Birman 1965; Dibblee 1966; Norris & Webb 1991) and could have been triggered by two different mechanisms: the creation of physical barriers between mountains and the creation of new habitats along altitudinal gradients. The major Pliocene glaciation that formed the North American ice sheets about 2.7 Ma ago, and ended a long warm period in the early to mid-Pleistocene (Cronin & Dowsett 1993), may have served as a climactic trigger for the speciation events beginning about 2.5–3 Ma ago (see Hewitt 1996).

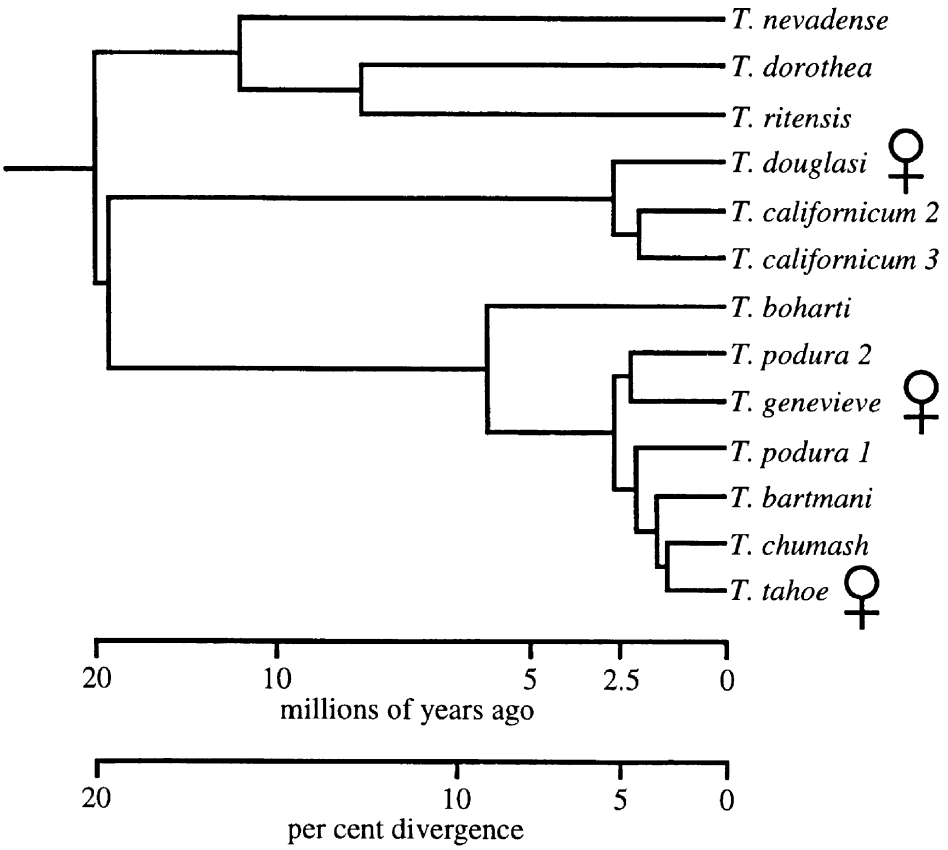

Figure 3. Tree inferred using maximum likelihood with an assumption of a molecular clock. Branch lengths are scaled in terms of expected numbers of substitutions, and the molecular clock calibration is based on the evolution of the same gene, *COI*, in other insects (Brower 1994; Juan *et al*. 1995, 1996). The timescale and divergence times should be interpreted as approximate. Parthenogenetic species are marked with a female symbol.

Although glaciers never reached southern California, the widespread cooling associated with this glacial period would be expected to have caused a downward altitudinal shift in the distribution of chaparral (e.g. during the Wisconsin glaciation in southern Nevada, juniper was at least 1200 m lower and white fir was 720 m lower than at present (Spaulding *et al*. 1983)). Thus, cooling would provide dispersal routes for *Timema* between mountain ranges, and interglacial periods would isolate populations on mountain tops. This hypothesis is consistent with the observation that the two *Timema* species adapted to lower-altitude chaparral plants, *T. podura* and *T. californicum*, currently have the widest distributions on different mountain ranges. Glaciation and concomitant shifts of host-plants to lower altitudes may also explain the 'transvalley leak' that allowed *T. genevieve* and its closest relatives, *T. podura* 2 and *T. tahoe*, to occur at opposite sides of the Central Valley.

According to a molecular clock for *COI*, the three parthenogenetic species of *Timema* each arose about 1.5–3 Ma ago. Each species retains a close sexual relative with a parapatric or allopatric and more southerly distribution, and for each species our phylogeny is consistent with an origin of the parthenogen from this relative. Two arguments might be raised against the inference that asexuality arose coincident with the inferred origins of the extant asexual lineages. First, if asexuality actually arose along these lineages more recently than 1.5–3 Ma ago, then either the asexual species drove their sexual progenitors extinct, or each lineage underwent a direct, complete transformation from sexuality to asexuality. The presence of unambiguously close sexual relatives (in two cases, relatives that are morphologically indistinguishable and on the same host-plant) for all three parthenogens indicates that both possibilities are highly implausible. Second, given our limited intraspecific sampling, more recent dates for the origin of parthenogenesis might be plausible if populations of more closely related sexual relatives exist but have not yet been sampled (Little & Hebert 1996). However, this argument is seriously weakened by our data on intraspecific variation in *T. californicum* and its related parthenogen *T. douglasi*: although intraspecific variation is high in *T. californicum* (0.3%, 4.5% and 5.2% for three specimens), each of these three specimens exhibits a similar level of divergence from *T. douglasi* (5.2%, 5.3% and 5.6%). The general similarity of the inferred ages of the three parthenogenetic species provides additional support for the accuracy of the inferences, as such similarity is unlikely to result from parallel extinctions of recent sexuals. Moreover, the similarity of the inferred ages is consistent with a common cause for the three origins, apparently associated with the cooling of North America about 2.7 Ma ago. These three asexual *Timema* species therefore appear to qualify as ancient parthenogens

(Judson & Normark 1996), whose further analysis should yield important clues to the maintenance of sex controversy. Determination of the mechanism of *Timema* parthenogenesis, and quantification of intraspecific divergence in nuclear and mtDNA within the parthenogenetic species (Chaplin & Hebert 1997), will allow more precise inference concerning the ages, origins, and causes of asexuality in this genus.

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